Remarks

The August 18, 2003 Official Action has been carefully reviewed. In view of the amendments submitted herewith and the following remarks, favorable reconsideration and allowance of this application are respectfully requested.

As a preliminary matter, Applicants are pleased to note that the Examiner has indicated that claims 1-3, 5, and 45-51 are in condition for allowance.

At page 2 of the Official Action, the Examiner has rejected claim 6 under 35 U.S.C. §101 as allegedly being drawn to unpatentable subject matter.

The Examiner has also rejected claim 4 under 35 U.S.C. §112, second paragraph as allegedly indefinite. Specifically, the Examiner alleges that the phrase "said MOAT-B transporter protein" lacks antecedent basis within the claim.

Claim 4 has also been rejected for allegedly failing to satisfy the written description requirement under 35 U.S.C. §112, first paragraph.

The Examiner has also maintained the rejection of claims 56-58 for allegedly failing to satisfy the enablement requirement under 35 U.S.C. §112, first paragraph.

The foregoing constitutes the entirety of the rejections raised in the August 18, 2003 Official Action. In light of the present claim amendments and the following remarks, each of the above-mentioned rejections is respectfully traversed.

In order to be completely responsive to the August 18, 2003 Official Action, Applicants have cancelled the previously withdrawn claims 8-44, 52-55, and 59 which are drawn to a nonelected invention. Applicants reserve the right to file one or more continuing applications, as provided under 35 U.S.C. §120, directed to the subject matter withdrawn from consideration in this application.

Please cancel claim 4.

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CLAIM 6 AS AMENDED MEETS THE REQUIREMENTS OF 35 U.S.C. §101

The Examiner has rejected claim 6 under §101 as allegedly drawn to unpatentable subject matter. Specifically, the Examiner contends the claim encompasses naturally occurring nucleic acid molecules. Applicants have, therefore, amended claim 6 to be drawn to an "isolated" nucleic acid molecule. Accordingly, Applicants respectfully request the withdrawal of the rejection of claim 6 under 35 U.S.C. §101.

THE REJECTIONS OF CLAIM 4 UNDER 35 U.S.C. §112, FIRST AND SECOND PARAGRAPHS HAVE BEEN RENDERED MOOT

The Examiner has rejected claim 4 under 35 U.S.C. §112, second paragraph for alleged indefiniteness. Specifically, the Examiner asserts that the phrase "said MOAT-B transporter protein" in claim 4 lacks antecedent basis.

Additionally, the Examiner has rejected claim 4 for allegedly failing the written description requirement under 35 U.S.C. §112, first paragraph. The Examiner contends that the specification fails to provide support for a nucleic acid comprising introns and exons which comprise SEQ ID NO: 1. Applicants submit that a skilled artisan equipped with the sequence of MOAT-B and the chromosomal location of the MOAT-B gene (see, for example, page 44, line 29 through page 45, line 8) would be readily able to identify a gene encoding for MOAT-B and the introns and exons present in the identified gene. However, in order to expedite prosecution of the instant application, Applicants have cancelled claim 4 thereby rendering moot the rejections of the claim under 35 U.S.C. §112, first and second paragraphs.

CLAIMS 56-58 MEET THE REQUIREMENTS OF 35 U.S.C. §112, FIRST PARAGRAPH

The Examiner has maintained the rejection of claims 56-58 under 35 U.S.C. §112, first paragraph as allegedly containing subject matter which was not described in the specification in such a way as to enable a skilled artisan to make and/or use the invention. Specifically, the Examiner contends that the similarity in amino acid sequence to a known transporter of anticancer drugs is insufficient to predict the biological function of MOAT-B. Furthermore, the Examiner asserts that a skilled artisan would be unable to determine if the test compounds were effecting MOAT-B activity or promoter activity.

Applicants respectfully submit that MOAT-B, as predicted by Applicants in the instant application, has been demonstrated to be capable of effectively effluxing anticancer agents by Lee et al. (J. Natl. Canc. Inst. (2000) 92:1934-40) which provides compelling evidence that the instantly claimed methods were fully enabled and described in the application as originally filed. Lee et al. disclose that the expression of MRP4 (MOAT-B) in transfected cells provides significant resistance to a four hour exposure to the anticancer agent methotrexate (see middle and right column of page 1936). Lee et al. also disclose, by a drug accumulation assay, that the expression of MRP4 (MOAT-B) in transfected cells conveys the ability to efflux radiolabeled anticancer agents (see left column page 1937 and Figure 2C). Notably, the methods employed to demonstrate the ability of MRP4 (MOAT-B) to efflux anticancer agents in Lee et al. are remarkably similar to the methods employed by Zaman et al. (PNAS (1994) 91:8822-26; copy enclosed), which is incorporated into the instant application by reference. Indeed, the drug accumulation assays of Zaman et al. and Lee et al. both entail incubating transfected cells with ^{3}H-labeled anticancer agents, washing with cold phosphate-

buffered saline, and detecting the location of the radiolabled anticancer agent by employing a liquid scintillation counter (see, e.g., page 8823 of Zaman et al. and page 1935 of Lee et al.). Applicants note, however, that the aforementioned assay is not intended to limit the invention to such an embodiment. Indeed, other assays, such as the cell survival assay (see, e.g., pages 1935-36 and Figure 2A of Lee et al. and page 8825 of Lee et al.), may be readily employed to ascertain the ability of a test compound to inhibit MOAT-mediated transport activity.

The Examiner also maintains the position that the claimed invention does not specify how to determine if the effect of the test compound on is MOAT-B activity or the promoter activity and, therefore, a skilled artisan would not know how to properly screen the compounds. Applicants submit, however, that it is a well settled premise in patent law that a patent need not teach, and preferably omits, what is well known in the art. <u>Lindemann Maschinenfabrik v. American Hoist and Derrick,</u> 221 USPQ 481, 489 (Fed. Cir. 1984). As noted in the MPEP at §2164, "detailed procedures for making and using the invention may not be necessary if the description of the invention itself is sufficient to permit those skilled in the art to make and use the invention." Applicants maintain that a skilled artisan, in the absence of specific instruction, would readily be able to devise a simple assay to ensure that the effect of the test compound is on MOAT-mediated transporter activity and not the particular promoter expressing MOAT. As noted in the Applicants response filed June 13, 2003 to the previous Official Action, such an assay could include linking a reporter gene to the promoter used to express MOAT, inserting the new construct into cells, and then testing for expression of the reporter gene in the presence and absence of the test compound to ensure the test compound had no effect on expression from the promoter. Clearly, such an assay could readily be designed by the skilled person and

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it cannot be reasonably maintained that such an assay comprises undue experimentation.

Moreover, Applicants submit that claim 56 cites "contacting said host cell with a compound **suspected** of inhibiting MOAT-mediated transported activity." Inasmuch as the compound is already suspected of inhibiting MOAT-mediated transporter **activity**, Applicants submit it would be highly unlikely for the compound to, additionally, adversely effect the promoter employed to express MOAT-B. Therefore, Applicants submit that the proposed scenario by the Examiner that the skilled artisan would not know if the compound is effecting MOAT activity or MOAT expression is (1) not a likely occurrence and (2) in the event it does occur, a skilled artisan would readily be able to perform simple experiments to distinguish between the two effects.

For all of the foregoing reasons, Applicants respectfully request the withdrawal of the rejection of claims 56-58 under 35 U.S.C. §112, first paragraph.

CONCLUSION

In view of the amendments presented herewith, and the foregoing remarks, it is respectfully urged that the objections and rejections set forth in the August 18, 2003 Official Action be withdrawn and that this application be passed to issue.

It is respectfully requested that the amendments presented herewith be entered in this application, since the amendments are primarily formal, rather than substantive in nature. This amendment is believed to clearly place the pending claims in condition for allowance. In any event, the claims as presently amended are believed to eliminate certain issues and better define other issues which would be raised on appeal, should an appeal be necessary in this case.

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In the event the Examiner is not persuaded as to the allowability of any claim, and it appears that any outstanding issues may be resolved through a telephone interview, the Examiner is requested to telephone the undersigned attorney at the phone number give below.

Respectfully submitted,

DANN, DORFMAN, HERRELL AND SKILLMAN

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Enclosures: Zaman et al. 1994
             Lee et al.

The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump

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ABSTRACT   The multidrug-resistance associated protein MRP is a 180- to 195-kDa membrane protein associated with resistance of human tumor cells to cytotoxic drugs. We have investigated how MRP confers drug resistance in SW-1573 human lung carcinoma cells by generating a subline stably transfected with an expression vector containing *MRP* cDNA. *MRP*-overexpressing SW-1573 cells are resistant to doxorubicin, daunorubicin, vincristine, VP-16, colchicine, and rhodamine 123, but not to 4′-(9-acridinylamino)methanesulfon-*m*-anisidide or taxol. The intracellular accumulation of drug (daunorubicin, vincristine, and VP-16) is decreased and the efflux of drug (daunorubicin) is increased in the transfectant. The decreased accumulation of daunorubicin is abolished by permeabilization of the plasma membrane with digitonin, showing that MRP can lower the intracellular daunorubicin level against a concentration gradient. Anti-MRP antisera predominantly stain the plasma membrane of *MRP*-overexpressing cells. We conclude that MRP is a plasma membrane drug-efflux pump.

Cells selected for resistance to a single cytotoxic drug may become crossresistant to a whole range of drugs with different structures and cellular targets. This phenomenon is called multidrug resistance (MDR). The classic form of MDR is due to an increased activity of P-glycoprotein (Pgp), encoded by the human *MDR1* gene (1–4) (standard gene symbol, *PGY1*). This large glycoprotein is located in the plasma membrane and can extrude a range of hydrophobic natural product drugs from the cell against a concentration gradient (1–5). An increase in Pgp activity can result in lowered intracellular drug concentration and, hence, in drug resistance.

Increased Pgp is not the only cause of MDR, however. Several cell lines selected for resistance do not contain increased amounts of Pgp but nevertheless are resistant to a broad range of natural-product drugs (6–9). In one of these non-Pgp MDR lines, the H69AR small-cell lung carcinoma (SCLC) line, Cole *et al.* (10) found amplification and increased expression of a novel gene, the *MRP* (MDR-associated protein) gene. Overexpression of *MRP* has since been observed in several other (11–14), but not all (11), non-Pgp MDR cell lines. Transfection of HeLa cells with an expression vector containing the *MRP* cDNA results in the acquisition of resistance to doxorubicin, vincristine, and VP-16, but not cisplatin (15). We show here that transfection of the *MRP* cDNA into human lung carcinoma cells also results in MDR.

These experiments prove that *MRP* is a drug-resistance gene, but they do not answer the question how MRP acts. As MRP belongs to the ABC superfamily of transporter proteins (10, 16), it could simply act like Pgp, as a plasma membrane

pump extruding drugs. Indeed, decreased drug accumulation has been reported for several non-Pgp MDR cell lines that were later found to overexpress *MRP* (7, 11–14). A major exception, however, is the MDR H69AR cell line in which the *MRP* gene was discovered (10). Drug accumulation was reported to be the same as in the parental cell line and this led Cole *et al.* (10, 17, 18) to consider other mechanisms than decreased drug accumulation for MRP action. Moreover, the subcellular location of MRP did not seem to be similar to that of a plasma membrane transporter such as Pgp. A 190-kDa protein detected in non-Pgp MDR cells and thought to be MRP was found mainly in the endoplasmic reticulum, rather than in the plasma membrane (13, 14).

To analyze the mechanism of action of MRP, we examined the effect of *MRP* overexpression on drug resistance and drug accumulation in SW-1573 lung carcinoma cells. In addition, we have raised antibodies against segments of MRP and used these to determine its main cellular location.

MATERIALS AND METHODS

Cell Lines. The drug-sensitive and doxorubicin-selected MDR sublines of the non-SCLC cell line SW-1573 and the SCLC line GLC4 have been described (7–9). The *MDR3* Pgp-expressing cell line V01V01 and the control cell line FVB-c are simian virus 40-immortalized mouse ear fibroblasts obtained from FVB mice transgenic for the human *MDR3* gene and normal FVB mice, respectively (19).

Vector Construction and Transfection. A DNA fragment containing the complete predicted *MRP* open reading frame (10, 15) plus 115 nt of 5′ and 800 nt of 3′ noncoding sequence was constructed in the expression vectors pJ3Ω (20) and pRc/RSV (Invitrogen). All cDNA fragments used for the assembly of the *MRP* cDNA were sequenced and the integrity of the *MRP* cDNA fragment in the resulting expression vectors, pJ3Ω-MRP and pRc/RSV-MRP (Fig. 1), was assessed by restriction enzyme mapping and DNA sequence analysis of the cloning junctions. Transfection of SW-1573/S1 cells with pRc/RSV-MRP DNA or African green monkey kidney COS-7 cells with pJ3Ω-MRP DNA followed the standard calcium phosphate precipitation technique (21). Stable transfectants in S1 were selected for 3 weeks in medium with G418 at 800 μg/ml and propagated for further analysis with G418 at 200 μg/ml. COS-7 cells were trypsinized 48 hr after transfection and analyzed by immunocytochemistry.

MRP Fusion Proteins and Immunization. Two fusion genes consisting of the gene for the *Escherichia coli* maltose-binding protein and two different segments of the *MRP* cDNA were constructed in the plasmid vector pMal-c (22).

Abbreviations: MDR, multidrug resistance (resistant); Pgp, P-glycoprotein; SCLC, small-cell lung cancer; pH$_i$, intracellular pH; *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide.

Medical Sciences: Zaman *et al.*

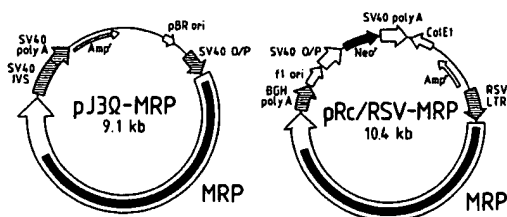*Proc. Natl. Acad. Sci. USA 91 (1994)*   8823

FIG. 1. *MRP* expression vectors. (*Left*) pJ3Ω-MRP, containing the *MRP* cDNA under control of simian virus 40 (SV40) regulatory elements. O/P, SV40 origin and early promoter; IVS, SV40 t-antigen intervening sequence; poly A, SV40 T-antigen polyadenylylation signal; Amp^r, β-lactamase gene; ori, origin of replication. (*Right*) pRc/RSV-MRP, containing the *MRP* cDNA under control of the Rous sarcoma virus long terminal repeat (RSV LTR). BGH poly A, bovine growth hormone gene polyadenylylation signal; Neo^r, neomycin-resistance gene.

The *MRP* sequences in the expression plasmids encoded aa 780–944 (fusion protein II) or 1294–1431 plus 1496–1531 (fusion protein III) of MRP. The fusion proteins were produced in *E. coli* JM101 and purified by amylose resin affinity chromatography (22). Guinea pigs (Sewall–Wright inbred strain) received sub- and intracutaneous injections of 200 μg of purified fusion protein II or III emulsified in Freund's complete adjuvant (Bacto, Detroit, MI). After 2 and 4 weeks booster injections with the same dose of immunogen in Freund's incomplete adjuvant were given intracutaneously, and after an additional 2 weeks the animals were bled.

Immunocytochemistry. Cytocentrifuge preparations of tumor cell lines were air-dried overnight, fixed for 10 min in acetone or 4% (vol/vol) paraformaldehyde in phosphate-buffered saline and incubated for 60 min with diluted antisera. Antibody binding was detected with a biotinylated mouse anti-guinea pig monoclonal antibody (Dako) and streptavidin-conjugated horseradish peroxidase (Zymed). The slides were developed with 3-amino-9-ethylcarbazole, counterstained with hematoxylin, and mounted with Aquamount.

Protein Blots. Total cell lysates were made by lysing harvested cells in 10 mM KCl/1.5 mM MgCl$_2$/10 mM Tris Cl, pH 7.4/0.5% (wt/vol) SDS supplemented with 1 mM phenylmethylsulfonyl fluoride, leupeptin (2 μg/ml), pepstatin (1 μg/ml), and aprotinin (2 μg/ml). DNA was sheared by sonication and samples containing 25 μg of protein were fractionated by SDS/7.5% PAGE and then transferred onto a nitrocellulose filter by electroblotting. Immunoreactivity of proteins with the antisera against the fusion proteins was visualized with peroxidase-conjugated rabbit anti-guinea pig immunoglobulins (Dako) and 3,3′-diaminobenzidine and 4-chloro-1-naphthol substrate. P-glycoproteins were detected by using the monoclonal antibody C219, rabbit anti-mouse IgG, and ^{125}I-labeled protein A. For glycosylation studies of MRP, membrane-enriched protein fractions were prepared and treated with *N*-glycanase (a mixture of endoglycosidase F and peptide:N-glycosidase F) (Boehringer Mannheim) (21).

Drug Accumulation. Steady-state accumulation of radiolabeled daunorubicin, vincristine, and VP-16 was measured according to Skovsgaard (23), as modified by Broxterman *et al.* (24). Cells in the logarithmic phase of growth (0.2 × 10^6 cells per ml) were incubated at 37°C with ^{3}H-labeled daunorubicin, vincristine, or VP-16. After 75 min ice-cold phosphate-buffered saline was added and after two cold washes radioactivity was determined by liquid scintillation counting. The cellular influx of daunorubicin was measured by monitoring the fluorescence decrease after addition of cells to daunorubicin with a fluorescence monitor (FluoroMax; Spex Industries, Metuchen, NJ) at excitation and emission wavelengths of 488 and 560 nm, respectively. Intracellular pH

(pH$_i$) was measured with 2'7'-bis(2-carboxyethyl)-5 (and 6)-carboxyfluorescein (BCECF) (25).

RESULTS

Cloning and DNA Sequence Analysis of *MRP* cDNA. *MRP* encodes an mRNA of ≈6.5 kb encompassing a continuous open reading frame of 1531 aa (10, 15). We have isolated a set of overlapping cDNA clones by hybridization screening of a cDNA library of adenovirus-transformed human retinal cells (RCA) and by reverse transcription–PCR of mRNA from the lung cancer cell line GLC4/ADR. Together these clones covered the complete predicted coding region of the *MRP* mRNA plus 137 nt of 5′ and 1137 nt of 3′ noncoding sequence; 916 nt of 3′ noncoding sequence were not published previously and have been deposited in the GenBank database (accession no. X78338). The cDNA clones obtained from the RCA cDNA library differ in a few nucleotides from the sequence published by Cole *et al.* (10): five of these are silent variations (T-1021 → C; T-1258 → C; T-1880 → C; C-1900 → T; A-1907 → C), whereas two lead to amino acid changes [C-546 → T (Thr-117 → Met); T-2250 → C (Leu-685 → Ser)].
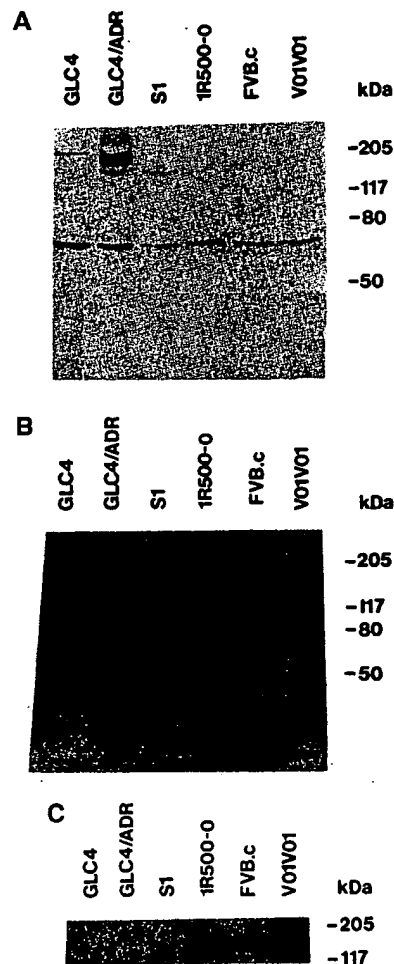

FIG. 2. Immunoblot analysis of total protein of cell lines with increased levels of MRP (GLC4/ADR), *MDR1* Pgp (1R500-0), or *MDR3* Pgp (V01V01) and of the corresponding parental cell lines (GLC4, S1, and FVB·c). Protein blots were incubated with antiserum against MRP fusion protein II (*A*), antiserum against MRP fusion protein III (*B*), or the Pgp-reactive monoclonal antibody C219 (*C*).

As the variation at aa 685 is in the highly conserved Walker A consensus motif in the amino-proximal nucleotide-binding domain (10, 16), we checked whether it was also present in cDNA from GLC4/ADR cells. This was the case. Since the reconstructed cDNA from the RCA cDNA clones encodes a functional MRP (see below), we conclude that the two amino acid changes do not impair the activity of MRP.

Generation of Anti-MRP Polyclonal Antisera. To examine the expression level and the cellular localization of MRP, we prepared polyclonal antisera against two bacterial fusion proteins containing different fragments of MRP. The first fusion protein contained the "linker" domain that connects the two halves of the protein (fusion protein II) (10). The second contained the carboxyl-terminal end and part of the predicted carboxyl-proximal nucleotide binding domain of MRP (fusion protein III) (10). The specificity of the antisera was determined on protein blots (Fig. 2) and on cytocentrifuge preparations of tumor cells and of African green monkey kidney COS-7 cells that transiently overexpress MRP (Fig. 3).

On immunoblots both antisera detected a protein of 190–195 kDa in total protein isolates of the GLC4/ADR cells, but not of the parental drug-sensitive cell line GLC4 (Fig. 2). This is in agreement with the massive increase of *MRP* mRNA and *MRP* gene-copy number in the GLC4/ADR cells, relative to the low basal *MRP* mRNA level in GLC4 (11). The sera did not crossreact with the human *MDR1* or *MDR3* Pgps (Fig. 2) but did crossreact with several small proteins not affected by the level of MRP and present in all cell lines. In addition, one of the antisera also reacted with a band of about 180 kDa in the two mouse cell lines (Fig. 2B). These bands did not precisely comigrate with Pgp and they might be mouse MRP.

In cytological preparations we saw strong staining with GLC4/ADR cells (Fig. 3 *B* and *C*) and other *MRP*-overexpressing cell lines, including the fibrosarcoma cell line HT1080/DR4 (12) and the leukemia cell line HL60/ADR (13) (data not shown), but not with the respective parental drug-sensitive cell lines (Fig. 3A). Staining was primarily over the plasma membrane. In most of the cells a dense spot near the nucleus was also seen, probably corresponding to the Golgi network. This may represent nonfunctional MRP or protein that is on its way to the plasma membrane. Both antisera showed the same staining pattern and specificity. We also saw strong reaction with COS-7 cells that were transiently transfected with the *MRP* expression vector pJ3Ω-MRP

(Figs. 1 and 3*G*), but not with "mock"-transfected cells (Fig. 3*F*).

Overexpression of MRP in SW-1573 Cells. To make stable transfectants in SW-1573 cells we cloned the *MRP* cDNA behind the Rous sarcoma virus promoter in the expression vector pRc/RSV (Fig. 1B). This vector also contains the bacterial *neo* gene allowing the selection of transfected cells with the neomycin analogue G418. pRc/RSV-MRP (Fig. 1B) was transfected into SW-1573/S1 cells, and after 3 weeks of selection with G418, 40 colonies were picked randomly and analyzed for *MRP* expression. Only 5 clones showed increased *MRP* mRNA, as measured by an RNase protection assay (11), indicating that in the other clones only the *neo* gene was correctly integrated. Of the 5 clones with increased *MRP* mRNA, 1, designated S1(MRP), also showed increased MRP in immunoblot analysis (Fig. 4A). In a second transfection experiment 1 of 30 G418-resistant transfectants analyzed overexpressed *MRP* [S1(MRP)-2, Fig. 4A]. Both transfectants with raised *MRP* mRNA levels were MDR, whereas G418-resistant clones that did not overexpress *MRP* were not (Table 1).

Glycosylation of MRP. MRPs from the transfectants comigrated with MRP present in S1 cells in SDS/PAGE and their mobility corresponded to ≈180 kDa (Fig. 4). MRPs from the resistant GLC4 and SW-1573 sublines all migrated slower (Fig. 4B). After treatment with N-glycanase, the two types of MRP both decreased in apparent mass to ≈170 kDa (Fig. 4C), in agreement with the 172 kDa predicted from the primary amino acid sequence of MRP. This suggests that the difference in mobility seen in untreated protein samples is due to a difference in glycosylation.

Location of MRP in the Cell. In cytocentrifuge preparations both anti-MRP antisera mainly stained the plasma membrane of S1(MRP) cells (Fig. 3E). There was also cytoplasmic staining, however, mostly homogeneous, but in some cells present as a concentric ring around the nucleus. Whether this ring represents the endoplasmic reticulum or the nuclear membrane remains to be determined. The relative fraction of MRP present in the plasma membrane of S1(MRP) seemed lower than in GLC4/ADR, suggesting that the routing of nascent MRP may differ in the two cell types. Untransfected S1 cells did not stain (Fig. 3D), confirming the specificity of the antisera for MRP.

Mechanism of MRP-Mediated MDR. The resistance spectrum of the *MRP*-overexpressing transfectants was compared with that of untransfected SW-1573/S1 cells in clono-
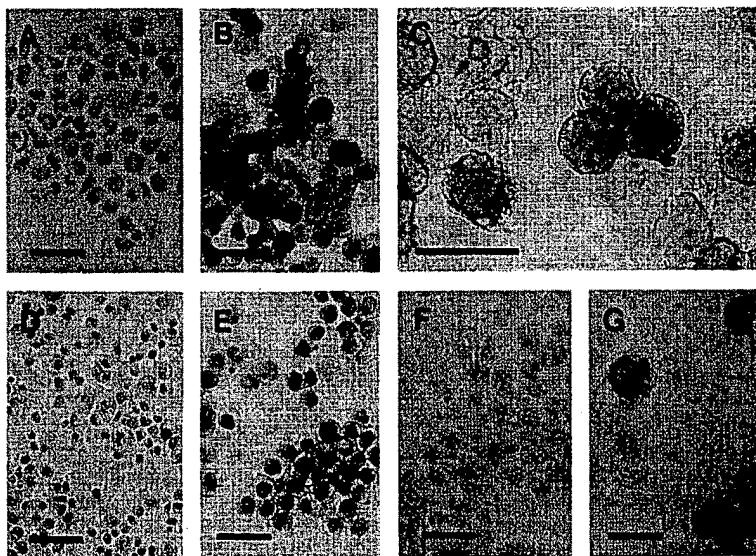

FIG. 3.   Immunochemical staining of cytocentrifuge preparations of *MRP*-overexpressing cells. (*A*) Drug-sensitive GLC4 cells. (*B* and *C*) Drug-resistant MRP-overexpressing GLC4/ADR cells (two different magnifications). (*D*) Drug-sensitive SW-1573/S1 cells. (*E*) S1(MRP), an *MRP*-overexpressing transfectant of S1. (*F*) COS-7 cells transfected with plasmid DNA lacking *MRP* cDNA sequences ("mock" transfection). (*G*) COS-7 cells transfected with pJ3Ω-MRP DNA (Fig. 1). The stained cells are the transiently transfected cells that highly overproduce MRP. In the COS-7 cells most MRP is in the cytoplasm. Therefore, the COS-7 expression system may not be suitable for functional studies on MRP. Slides were stained with antiserum against MRP fusion protein III. Staining with antiserum against fusion protein II gave similar results. (Bars = 50 μM.)
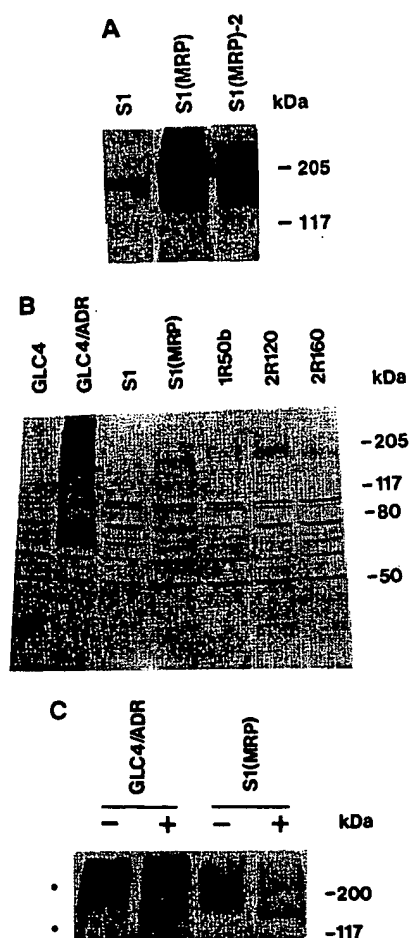
FIG. 4. Immunoblot analysis of MRP in untransfected S1 cells and *MRP*-overexpressing transfectants [S1(MRP) and S1(MRP)-2] (*A*) and drug-sensitive (GLC4 and S1) and drug-resistant lung cancer cell lines [GLC4/ADR, S1(MRP), 1R50b, 2R120, and 2R160] (*B*) and of MRP glycosylation in GLC4/ADR and S1(MRP) cells (*C*). +, Membrane-enriched protein fractions treated with *N*-glycanase; −, untreated control samples. Migration of protein markers of 117 and 200 kDa is indicated with dots at left and with bars at right. Blots were stained with the anti-MRP monoclonal antibody MRPr1 (M.J.F., R.J.S., and G.J.R.Z., unpublished data) (*A*) or with polyclonal antiserum against MRP fusion protein III (*B* and *C*).

genic survival assays. The results for S1(MRP) are summarized in Table 1. Overexpression of *MRP* in SW-1573 cells increases resistance to doxorubicin, vincristine, and VP-16 (Table 1), as also reported by Grant *et al.* (15) for HeLa cells. In addition we found increased resistance to daunorubicin, colchicine, and rhodamine 123, but not to *m*-AMSA and taxol (Table 1). S1(MRP)-2 was about 2-fold resistant to doxorubicin, vincristine, and VP-16, and not to *m*-AMSA, thus confirming the results for S1(MRP).

Resistance was associated with a decreased steady-state accumulation of drug (Table 2). The mechanism of decreased drug accumulation in S1(MRP) was examined in detail for daunorubicin. As this is a weak base, its accumulation could be decreased by an increase in pH_i (26). This was not the case, as we found a pH_i of 7.27 ± 0.13 (mean $\pm$ SD; $n = 3$) in S1 cells and a pH_i of 7.30 ± 0.10 in S1(MRP) cells. A decrease in drug accumulation could also be caused by a decrease in cellular binding sites. To test this possibility, we

selectively permeabilized the cellular plasma membrane with digitonin (see ref. 25). Upon addition of digitonin, the accumulation of daunorubicin in S1(MRP) cells became equal to that in S1 cells (Table 2), showing that the accumulation deficit in S1(MRP) was not due to fewer cellular binding sites. This was confirmed by an experiment where the uptake of daunorubicin into S1 and S1(MRP) was followed in time and compared with the uptake into the 2R120 subline (Fig. 5). The fluorescence decrease represents mainly quenching of daunorubicin by binding to the DNA (27). The accumulation of daunorubicin was lower in S1(MRP) and 2R120 cells than in S1 cells, in agreement with Table 2. Upon addition of digitonin, S1(MRP) and 2R120 showed a large increase in DNA binding (Fig. 5). These results demonstrate that the decreased accumulation of daunorubicin in the *MRP*-overexpressing cells was not due to a passive redistribution of drug, but due to an active process. When we therefore tested daunorubicin efflux from transfected and control cells; the efflux of daunorubicin was faster from S1(MRP) than from S1 cells (Fig. 6). We conclude that MRP can mediate the active extrusion of daunorubicin from cells.

DISCUSSION

Our results show that MRP is remarkably similar to the drug-transporting Pgps in its mode of action. (*i*) Like Pgp, MRP can cause resistance to a range of hydrophobic drugs. (*ii*) MRP is predominantly located in the plasma membrane. (*iii*) MRP can decrease drug accumulation in the cell and this decrease is abolished by permeabilization of the plasma membrane. (*iv*) MRP can increase the efflux of drugs from cells. We therefore think that MRP acts as a drug pump, like Pgp, extruding hydrophobic compounds from cells against a concentration gradient. Presumably the two ATP-binding motifs in MRP allow the protein, just like Pgp, to use ATP hydrolysis for active transport.

The drug-resistance spectra associated with *MRP* and *MDR1* overexpression are remarkably similar, given the

Table 1. Resistance spectrum of the *MRP* transfectant S1(MRP)

Drug	Relative resistance
Doxorubicin	2.7 ± 0.4
Daunorubicin	3.2 ± 0.8
Vincristine	5.3 ± 1.3
VP-16	4.9 ± 1.1
m-AMSA*	1.1 ± 0.1
Colchicine	3.6 ± 0.1
Rhodamine 123	4.1 ± 0.3
Taxol	1.0 ± 0.1

Resistance was determined in clonogenic survival assays (8). Relative resistance = IC_{50} S1(MRP)/IC_{50} S1 (IC_{50}, inhibitory concentration where 50% of the cells survive). The values are the means $\pm$ SD of at least three experiments, each performed in duplicate. *4′-(9-Acridinylamino)methanesulfon-*m*-anisidide.

Table 2. Accumulation of daunorubicin (0.5 μM, 75 min), vincristine (0.5 μM, 75 min) and VP-16 (5 μM, 75 min) in untransfected S1 cells and the *MRP* transfectant S1(MRP)

	pmol per 10^6 cells			
	Daunorubicin*			
Cell line	− digitonin	+ digitonin†	Vincristine*	VP-16‡
S1	367 ± 30	292 ± 39	28.8 ± 3.6	13.0
S1(MRP)	211 ± 22	295 ± 25	16.1 ± 4.4	5.5

*Mean $\pm$ SD of four independent experiments, each performed in quadruplicate.
†Digitonin (20 μM) was added 5 min before the end of the incubation.
‡Mean of two independent experiments, each performed in quadruplicate. Variation was <2 pmol per 10^6 cells.
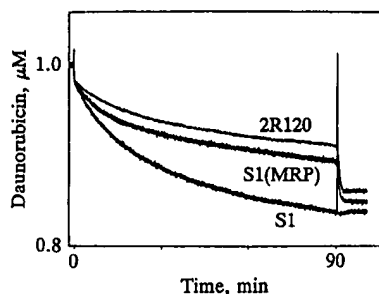
FIG. 5. Time course of daunorubicin uptake by SW-1573 cell lines. One million S1, S1(MRP), or 2R120 cells were added to 2.5 ml of medium containing 1 μM daunorubicin. The decrease in fluorescence due to increased influx of daunorubicin and binding to DNA was recorded. After 90 min of incubation, digitonin (20 μM) was added to the medium.

large difference in sequence between MRP and the *MDR1*-encoded Pgp (10). Nevertheless, there are also important differences between MRP and *MDR1* Pgp in the drugs that they transport or interact with. We found no resistance to taxol in lung carcinoma cells transfected with *MRP* cDNA (Table 1), whereas mouse bone marrow cells transgenic for *MDR1* are highly resistant (28). In a preliminary search for reversal agents of MRP-mediated MDR, we found that the decreased accumulation of daunorubicin in the *MRP* transfectant was not affected by cyclosporin A, an effective reversal agent of Pgp-mediated MDR. In contrast, the isoflavinoid genistein, a drug that does not inhibit *MDR1* Pgp-mediated drug transport (29), slightly reduced the decreased drug accumulation in the *MRP* transfectant. This suggests that genistein is a modulator of MRP-mediated MDR. Since genistein is too toxic for use in patients and even too toxic to use as a convenient reversal agent in drug resistance tests (29), less toxic analogues of genistein that also act on MRP are needed.

MRP in the non-Pgp MDR cell line SW-1573/2R120 is not further increased in the Pgp-overexpressing cell line 2R160 (Fig. 4B). As this cell line was derived from 2R120 by further selection on doxorubicin (9), the extent to which *MRP* overexpression is tolerated in SW-1573 cells may be limited to the level reached in 2R120. Consequently, MRP-mediated MDR may play a role only in low-level MDR in SW-1573 cells and possibly in other cells as well.

There is little information on the contribution of increased levels of MRP to drug resistance of human cancers. High levels of *MRP* mRNA were found in leukemic cells of a high
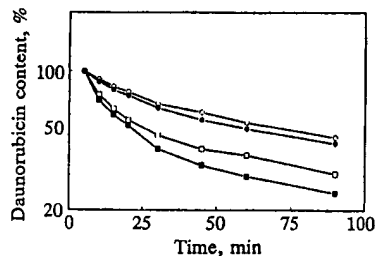

FIG. 6. Normalized cellular efflux of [G-³H]daunorubicin from SW-1573/S1 cells (circles) or S1(MRP) cells (squares). Cells were loaded with daunorubicin (0.5 μM) for 60 min and suspended in daunorubicin-free medium. At intervals thereafter, the amount of cellular daunorubicin was measured. Two separate experiments (closed and open symbols) are shown.

percentage of patients with chronic lymphocytic leukemia, but there was no relation to prior chemotherapy or treatment outcome (30). The phenotype delineated in this paper, the anti-MRP antisera, and the methods used here to demonstrate reduced drug accumulation should help t determine the role of MRP-mediated MDR in clinical drug resistance.

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Analysis of the MRP4 Drug Resistance Profile in Transfected NIH3T3 Cells

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Background: Multidrug resistance-associated protein (MRP) 1 and canalicular multispecific organic anion transporter (cMOAT or MRP2) are adenosine triphosphate-binding cassette transporters that confer resistance to anticancer agents. In addition to these two transporters, there are at least four other human MRP subfamily members (MRP3 through MRP6). We and others reported previously that MRP3 is capable of conferring resistance to certain anticancer agents. In this study, we investigated whether MRP4 (MOAT-B), whose transcript accumulates to the highest levels in prostate tissue, has the capacity to confer drug resistance. *Methods:* MRP4-transfected NIH3T3 cells were generated, and their drug sensitivity was analyzed. The subcellular localization of MRP4 was assessed by immunohistochemical analysis in transfected cells and in prostate tissue. Statistical tests were two-sided. *Results:* MRP4 was detected as a 170-kd protein that was localized in the plasma membrane and cytoplasm of transfected cells. The MRP4 transfectants displayed 5.5-fold increased resistance to methotrexate in short-term drug-exposure assays ($P = .022$) and exhibited decreased cellular accumulation of this agent at 4 hours ($P = .006$) and 24 hours ($P < .001$). In continuous-exposure assays, however, the MRP4 transfectants did not display increased resistance for either methotrexate or natural product cytotoxic agents (anthracyclines, etoposide, vinca alkaloids, and paclitaxel [Taxol]). However, the transfectants did show increased resistance (2.3-fold) for the anti-acquired immunodeficiency syndrome nucleoside analogue 9-(2-phosphonylmethoxyethyl)adenine (PMEA) ($P = .022$) in continuous-exposure assays. Consistent with MRP4's plasma membrane localization in transfected cells, analysis of prostate tissue showed that MRP4 protein was localized primarily in the basolateral plasma membranes of tubuloacinar cells. *Conclusions:* These results indicate that MRP4 confers resistance to sh rt-term methotrexate and continuous PMEA treatment. Given its structure, drug resistance profile and subcellular localization, MRP4 probably functi ns as an amphipathic anion efflux pump whose substrate range includes glutamate and phosphate conjugates. [J Natl Cancer Inst 2000;92:1934-40]

P-glycoprotein (Pgp), a plasma membrane efflux pump that functions to extrude diverse natural product drugs from the cell, has served as a paradigm for the role of adenosine triphosphate-binding cassette transporters in resistance to anticancer agents and for development of the idea that inhibitors of plasma membrane pumps may be useful for overcoming clinical resistance *(1)*. Following the identification of Pgp, studies of an anthracycline-resistant HL60 cell line that displays a drug accumulation defect but does not overexpress PgP led to the identification, using peptide antisera directed toward conserved epitopes of a Pgp nucleotide-binding fold, of a distinct 190-kd resistance-associated protein *(2)*. The complementary DNA (cDNA) that encodes this 190-kd protein, multidrug resistance-associated protein (MRP) 1, has been isolated from an anthracycline-resistant lung cancer cell line *(3)* and shown in transfection studies to have the capacity to confer a multidrug resistance phenotype *(4,5)*. While the MRP1 multidrug-resistant phenotype overlaps with that of Pgp *(6-8)*, its substrate selectivity is distinct. In contrast to Pgp, MRP1 is an organic anion transporter whose substrates include glutathione and glucuronate conjugates *(9-12)*. An organic anion transporter related to MRP1, the canalicular multispecific organic anion transporter (cMOAT or MRP2), has been isolated *(13-15)*, and experiments using the recombinant protein have demonstrated that it confers resistance to some anticancer agents *(16,17)* and that it shares the substrate selectivity of MRP1 with regard to glutathione and glucuronate conjugates *(17-21)*.

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See "Notes" following "References."

In addition to MRP1 and MRP2, four other MRP subfamily transporters have been described in humans (22–30), and two have been described in the rat (31). On the basis of degree of amino acid identity and predicted topologies of the full-length proteins, we discerned two groups: the first group consists of MRP1, MRP2, MRP3, and MRP6, and the second group consists of MRP4 and MRP5 (24). The first group is distinguished by a high degree of amino acid identity with MRP1 (45%–58% overall; 61%–74% nucleotide-binding folds) as well as having an N-terminal hydrophobic extension that constitutes a third membrane-spanning domain. The second group of transporters is less related to MRP1 in terms of amino acid identity (36%–39% overall; 57%–62% nucleotide-binding folds) and, in addition, has only two membrane-spanning domains.

In view of the capacity of MRP1 and MRP2 to confer resistance to anticancer agents, defining the drug resistance profiles of the more recently described members of the MRP subfamily is of interest. Analyses of the phenotypes associated with expression of the cloned cDNAs of two of these transporters, MRP3 and MRP5, have been reported. Our laboratory (32) reported that expression of MRP3 in HEK293 cells confered resistance to etoposide, vincristine, and methotrexate, and another laboratory (33) reported that MRP3-transduced ovarian carcinoma cells exhibited resistance to etoposide and methotrexate. In the case of MRP5, resistance to several anticancer agents was not observed in transfected HEK293 cells (34). The anticancer drug resistance phenotypes of two MRP subfamily members, MRP4 and MRP6, have yet to be defined in transfection studies. In this study, we report the first analysis of the capacity of the cloned MRP4 cDNA to confer drug resistance in transfected cells.

MATERIALS AND METHODS

Plasmid Constructs and Transfection of NIH3T3 Cells

A cDNA fragment encoding the 1325 amino acid MRP4 coding sequence (22) was assembled, and the nucleotides preceding the initiation site were modified from GCAAGATG to CCACCATG by use of polymerase chain reaction methodology to better conform to the Kozak consensus sequence. The cDNA was inserted into the insect expression vector pVL1393 to create pVL1393-MRP4 and into the retroviral-based expression vector pSRαMSVTKneo

(35) to create pSRα-MRP4. Insect cells were transfected with pVL1393-MRP4 according to the manufacturer's protocol (Invitrogen Corp., Carlsbad, CA), and NIH3T3 cells were transfected as described previously (6). Individual G418-resistant colonies were isolated by the cloning cylinder technique and expanded for further analysis. Clone NIH3T3/MRP4-3 cells were used in this study.

Generation of MRP4 Monoclonal Antibody and Immunoblotting

A cDNA fragment encoding amino acids 45–129 of MRP4 was inserted downstream of the glutathione S-transferase coding sequence in the pGEX-2T prokaryotic expression vector (Pharmacia Biotech, Inc., Piscataway, NJ). Inclusion bodies were prepared from induced bacteria, and the insoluble fusion protein was isolated by electroclution from preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels. Immunization of BALB/c mice with the recombinant protein, splenic fusion, and enzyme-linked immunosorbent assays was performed as described previously (36). For enzyme-linked immunosorbent assays of hybridoma supernatants, lysates of insect cells infected with an MRP4 baculovirus were used to coat 96-well dishes. (Expression of MRP4 in insect cells was confirmed by immunoblotting with a polyclonal MRP4 antibody generated against amino acids 577–706 of MRP4.) Ascites fluid generated by injection of hybridoma cells into the peritoneal cavity of severe combined immunodeficiency mice was used in the experiments that required MRP4 monoclonal antibody.

Analysis of Drug Sensitivity and Methotrexate Accumulation

Drug sensitivity was analyzed by use of a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt/phenazine methosulfate (MTS/PMS) microtiter plate assay (CellTiter 96 Cell Proliferation Assay; Promega Corp., Madison, WI). Control and MRP4-transfected cells were seeded in triplicate at 1000 per well in 96-well dishes in Dulbecco's modified Eagle medium supplemented with 10% calf serum. The next day, drugs at various dilutions were added to the growth medium. Growth assays were performed after 72 hours of growth in the presence of drug. For short methotrexate-exposure assays, methotrexate at various concentrations was added the day after seeding, and 4 hours later, the medium was removed. The cells were washed two times with complete medium, and drug-free medium was added. Seventy-two hours after methotrexate was added, the growth assays were performed using the MTS/PMS assay. Daunorubicin, doxorubicin, etoposide, vincristine, paclitaxel (Taxol), and potassium antimony tartrate were purchased from Sigma Chemical Co. (St. Louis, MO); methotrexate was purchased from Bedford Laboratory (Bedford, OH); and cisplatin was purchased from Bristol-Myers Squibb (Princeton, NJ). 9-(2-Phosphonylmethoxyethyl)adenine (PMEA) and 2′,3′-dideoxycytidine (ddC) were from Gilead (Forest City, CA) and Hoffmann-La Roche Inc. (Nutley, NJ), respectively. 2′-Deoxy-3′-thiacytidine (3TC) and 3′-azido-3′-deoxythymidine (AZT) were from GLAXO, Inc. (Research Triangle

Park, NC), and 2′,2′-didehydro-3′-deoxythymidine (d4T) was from Bristol-Myers Squibb.

For methotrexate accumulation experiments, approximately 1×10^6 control and MRP4-transfected cells were seeded in triplicate in 75-cm^2 tissue culture flasks; the next day, [^{3}H]methotrexate (Moravek, Brea, CA) was added to a concentration of $1 \mu M$ (0.5μCi/mmol). At 4 and 24 hours, the cells were washed with cold phosphate-buffered saline (PBS) and trypsinized. An aliquot of cells was used to analyze cell number, and radioactivity was measured by use of a liquid scintillation counter.

Statistical Methods

Cellular survival curves were obtained by simultaneously measuring percent survival for control and MRP4-transfected cells over a range of drug concentrations. For each drug concentration, the plotted percent survival represents the average of triplicate determinations (see Fig. 2 for examples). From a statistical perspective, the sample point in an experiment is defined as the 50% kill dose (IC_{50}) of an agent. The IC_{50} is obtained by empirically fitting a straight line in the region of the IC_{50} and ascertaining this value by graphic means. Since the IC_{50} of both control and MRP4-transfected cells are obtained from the same experiment, these measurements are treated as being paired. The nonparametric two-tailed Wilcoxon test was used to make inferences about the difference between the IC_{50} of the control and MRP4-transfected cells.

Mixed effects analysis of variance was used to analyze the methotrexate accumulation data. Cell line and time were modeled as fixed effects, while the triplicate measurements were accommodated as random effects. A posteriori multiple comparisons were performed by use of Scheffe's adjustment. The statistical analysis was performed by use of standard computer software statistical packages (Minitab Statistical Software; SAS Institute, Cary, NC).

Immunoblotting and Immunohistochemistry

Immunoblot analysis of crude membrane fractions was performed as described previously (32). MRP4 monoclonal antibody was used at 1:100, and antibody reactivity was detected by chemiluminescence (Amersham Life Science Inc., Arlington Heights, IL).

For immunocytochemical analysis, 1×10^4 cells were seeded on coverslips in six-well dishes. The cells were allowed to grow for 2 days, and the coverslips were washed two times with cold PBS and fixed in cold 4% paraformaldehyde/PBS for 10 minutes at room temperature. The coverslips were washed three times with PBS, and the cells were permeabilized with 0.02% Triton X in PBS for 10 minutes at room temperature. Blocking was accomplished with 10% goat serum, and the cells were incubated with MRP4 monoclonal antibody (1:100) in 3% bovine serum albumin for 1 hour at room temperature. The cells were washed five times in 0.02% Triton X in PBS treated with antimouse-rhodamine-X-conjugated goat antibody (1:200) (from The Jackson Laboratory, West Grove, PA) for 1 hour at room temperature and then washed five times in PBS. Imaging was accomplished by confocal microscopy.

Surgical specimens of human prostate fixed in 10% phosphate-buffered formaldehyde and embedded in paraffin were used in immunohistochemical experiments. Antigen retrieval was accomplished by boiling deparaffinized 5-μm-thick paraffin sections for 10 minutes in distilled water by use of a 750-W microwave oven at low setting. After preincubation with goat serum and peroxidase blocking, the sections were incubated overnight at 4 °C with MRP4 monoclonal antibody (1 : 100). After washing with PBS for 10 minutes, the immunohistochemical reaction was accomplished by use of a avidin–biotin-peroxidase kit (Vectastain Elite; Vector Laboratories, Inc., Burlingame, CA) with diaminobenzidine as chromogen.

RESULTS

Generation of MRP4-Transfected NIH3T3 Cells and Drug-Sensitivity Analysis

The integrity of the MRP4 coding sequence and the specificity of MRP4 immunologic reagents were tested by expressing the full-length recombinant protein in insect cells. As shown in Fig. 1 (lane 2), MRP4 was readily detected by use of MRP4 monoclonal antibody in insect cells infected with an MRP4 baculovirus. The protein migrated with an apparent molecular mass of 150 kd, in good agreement with its calculated molecular mass of 149563 d. Having established that our immunologic reagents could detect the recombinant MRP4 protein, we next transfected NIH3T3 fibroblasts with pSRαMRP4, a retroviral-based expression vector harboring the MRP4 coding sequence, or the parental vector. Colonies were selected for growth in the presence of G418, resistance to which is conferred

by the aminoglycoside 3'-phosphotransferase gene of the vector. Membranes were prepared from G418-resistant colonies, and expression of MRP4 was examined by immunoblot analysis. The expression of MRP4 in NIH3T3/MRP4-3, the G418-resistant colony in which the protein was most readily detected, is shown in Fig. 1 (lane 4). MRP4 migrated with an apparent molecular mass of approximately 170 kd. As expected for a glycosylated-transmembrane protein, the apparent molecular mass of MRP4 expressed in NIH3T3 cells was higher than that of the recombinant protein expressed in insect cells, which are only partially glycosylation competent.

The influence of MRP4 overexpression on the drug sensitivity of NIH3T3/MRP4-3 was analyzed by use of a colorimetric assay. Various structurally diverse anticancer chemotherapeutic agents were examined. Statistically significant resistance was observed for methotrexate when NIH3T3/MRP4-3 was analyzed in

experiments in which methotrexate exposure was limited to the first 4 hours of a 3-day growth assay (Fig. 2, A). In this short-exposure assay, NIH3T3/MRP4-3 displayed an average of 5.5-fold resistance relative to the control-transfected cell line (P = .022; Table 1). In the standard 3-day continuous-exposure growth assays, increased resistance was not observed for methotrexate, cisplatin, or several lipophilic cytotoxic drugs, including anthracyclines, etoposide, vincristine, and paclitaxel. Resistance to heavy metals was also examined because MRP1 has been associated with resistance to this class of compounds. As shown in Table 1, increased resistance was not detected for several heavy metals, including cadmium chloride, potassium antimony tartrate, and sodium meta-arsenate.

Several anti-acquired immunodeficiency syndrome (AIDS) drugs that either are organic anions or are converted in the cell to organic anions by phosphorylation were also tested. Statistically significantly
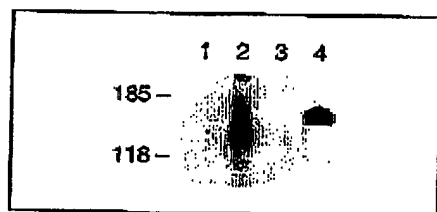

Fig. 1. Immunoblot detection of multidrug resistance-associated protein (MRP) 4 in infected insect cells (sf9) and transfected NIH3T3 cells. Cellular lysates prepared from sf9 cells (2μg) or membrane preparations of parental vector-transfected NIH3T3 cells and NIH3T3/MRP4-3 cells (30μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and MRP4 was detected by immunoblotting with anti-MRP4 monoclonal antibody. Lane 1—Sf9 cells infected with a control baculovirus; lane 2—Sf9 cells infected with an MRP4 baculovirus; lane 3—NIH3T3 cells transfected with the parental pSRα vector; and lane 4—NIH3T3/MRP4-3 cells. The locations of protein molecular weight markers are shown to the left.
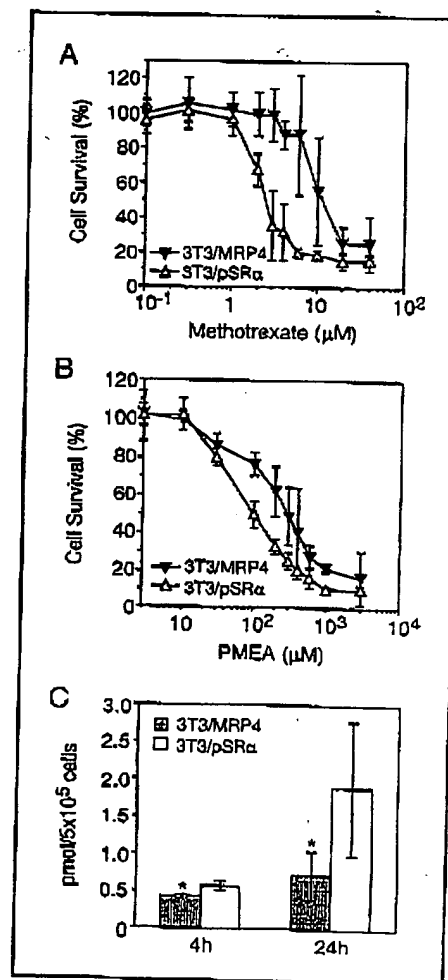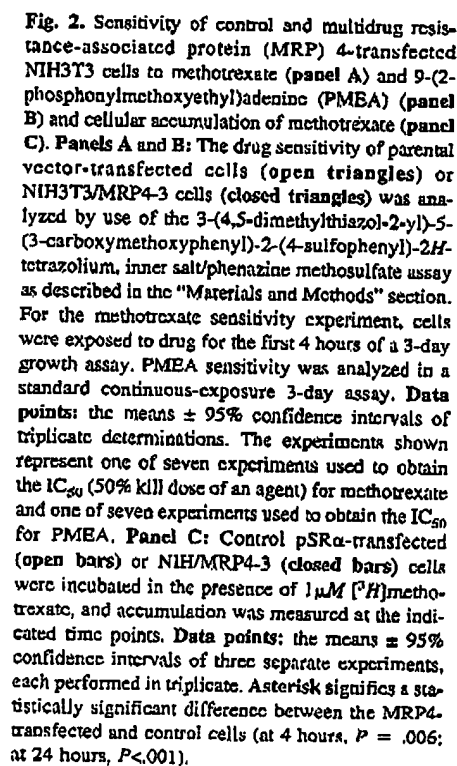
Fig. 2. Sensitivity of control and multidrug resistance-associated protein (MRP) 4-transfected NIH3T3 cells to methotrexate (panel A) and 9-(2-phosphonylmethoxyethyl)adenine (PMEA) (panel B) and cellular accumulation of methotrexate (panel C). Panels A and B: The drug sensitivity of parental vector-transfected cells (open triangles) or NIH3T3/MRP4-3 cells (closed triangles) was analyzed by use of the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt/phenazine methosulfate assay as described in the "Materials and Methods" section. For the methotrexate sensitivity experiment, cells were exposed to drug for the first 4 hours of a 3-day growth assay. PMEA sensitivity was analyzed in a standard continuous-exposure 3-day assay. Data points: the means ± 95% confidence intervals of triplicate determinations. The experiments shown represent one of seven experiments used to obtain the IC$_{50}$ (50% kill dose of an agent) for methotrexate and one of seven experiments used to obtain the IC$_{50}$ for PMEA. Panel C: Control pSRα-transfected (open bars) or NIH/MRP4-3 (closed bars) cells were incubated in the presence of 1μM [^{3}H]methotrexate, and accumulation was measured at the indicated time points. Data points: the means ± 95% confidence intervals of three separate experiments, each performed in triplicate. Asterisk signifies a statistically significant difference between the MRP4-transfected and control cells (at 4 hours, P = .006; at 24 hours, P<.001).

Table 1. Drug sensitivity of NIH3T3/MRP4-3 cells

Drug	IC50* μM		N†	Fold resistance‡
	NIH3T3/pSRα	NIH3T3/MRP4-3		
Daunorubicin	0.38 ± 0.11	0.40 ± 0.46	4	1.06
Doxorubicin	0.29 ± 0.11	0.33 ± 0.25	4	1.14
Etoposide	2.33 ± 0.49	2.61 ± 1.86	5	1.12
Vincristine	0.021 ± 0.008	0.022 ± 0.014	4	1.05
Paclitaxel (Taxol)	0.081 ± 0.074	0.090 ± 0.078	5	1.11
Methotrexate	0.059 ± 0.030	0.066 ± 0.041	4	1.13
Methotrexate, 4-h exposure	5.61 ± 4.48	31.0 ± 33.0	7	5.52§
Cisplatin	208 ± 56.4	247 ± 132	6	1.19
Cadmium chloride	1.82 ± 0.85	2.30 ± 1.24	5	1.27
Potassium antimony tartrate	62.7 ± 16.6	52.0 ± 43.9	3	0.83
Sodium meta-arsenite	18.5 ± 3.22	20.9 ± 5.41	5	1.13
PMEA	138.6 ± 37.6	315.7 ± 63.4	7	2.28§

*Drug sensitivities were analyzed by use of a 3-day colorimetric assay in which cells were continuously exposed to the indicated agents. In addition to the standard 3-day cytotoxicity assay, methotrexate sensitivity was also analyzed in a short-exposure assay in which cells were exposed to drug for only the first 4 hours of a 3-day growth assay. IC50 is the drug concentration that inhibited cell growth by 50%. Means ± 95% confidence intervals are provided.

†Number of individual measurements of the IC50 of NIH3T3/pSRα and NIH3T3/MRP4-3. Each measurement was performed in triplicate.

‡IC50 of NIH3T3/MRP4-3 ÷ IC50 of NIH3T3/pSRα.

§Statistically significantly different from the control transfectant as assessed by the nonparametric two-tailed Wilcoxon test: P = .022 for methotrexate; P = .022 for 9-(2-phosphonylmethoxyethyl)adenine (PMEA).

increased resistance was detected for the nucleoside phosphate analogue PMEA, for which an average of 2.3-fold resistance was observed (P = .022; Fig. 2, B; Table 1). However, increased resistance was not detected for several other nucleoside analogues, including AZT, 3TC, ddC, and d4T (data not shown).

Accumulation of Methotrexate

To examine the possibility that MRP4 influences the cellular kinetics of methotrexate, the accumulation of this agent was examined. Since methotrexate undergoes polyglutamylation in the cell, accumulation of radiolabeled drug reflects both free and polyglutamylated forms. As shown in Fig. 2, C, NIH3T3/MRP4-3-transfectant cells exhibited an accumulation deficit compared with the control-transfected cells. At 4 hours after incubation with radiolabeled methotrexate, accumulation in NIH3T3/MRP4-3 cells was 75% of the value observed in the control-transfected cells (P = .006). At 24 hours, the accumulation deficit was more pronounced, reaching 38% of the value observed in control-transfected cells (P<.001).

Localization of MRP4 Protein in NIH3T3/MRP4-3 Cells and Prostate

Reduced accumulation of methotrexate suggested that MRP4 might function as a plasma membrane efflux pump. To gain further insight into MRP4 function, its subcellular localization was examined. With the use of indirect immunofluorescence and confocal microscopy, subcellular localization was first analyzed in MRP4-transfected cells. As shown in the composite (Fig. 3, A and D, top) and mid-nuclear (Fig. 3, B and E, top) images, MRP4 was detected in both the cytoplasm and the plasma membranes of NIH3T3/MRP4-3 cells, whereas background staining was observed in NIH3T3 cells transfected with the parental vector (Fig. 3, C and E, top). A similar staining pattern was observed by use of a polyclonal MRP4 antibody raised against amino acids 577–706 of the protein (data not shown).

To determine whether the subcellular localization observed in transfected cells accurately reflected physiologic localization in tissues, we next examined MRP4 expression in the prostate, the tissue in which we previously detected the highest levels of MRP4 transcript by RNA blot analysis (22). As shown in Fig. 3, A (bottom), MRP4 was detected predominantly in the basal cells of the prostatic glandular epithelium, in which it was localized in the basal and lateral portions of the plasma membrane. Fig. 3, B (bottom), shows that, in addition to basolateral plasma membrane staining, MRP4 was also detected in the basolateral perinuclear cytoplasmic region of the basal cells. Staining was patchy, with some glands strongly stained and other glands weakly

stained within the same section. A similar staining pattern was observed by use of an MRP4 polyclonal antibody (data not shown). These immunologic reagents were not capable of detecting MRP4 protein in other tissues examined.

DISCUSSION

This study extends our understanding of the anticancer drug resistance activities of MRP subfamily members by demonstrating that MRP4, like MRP1–3 (32,33, 37), confers resistance to the antimetabolite methotrexate. As with MRP1–3, the capacity of MRP4 to confer resistance to this agent is pronounced in short-term drug-exposure assays but modest or undetectable in continuous-exposure assays. While MRP4 confers methotrexate resistance, its activity is less than that observed for MRP1 through MRP3, for which resistance levels of 21-fold to 78-fold were reported (33,37). This may reflect differences in protein-expression levels in the transfected cells, in the relative amounts of protein sorted to the plasma membrane, or in the recipient cell lines. However, we favor the idea that methotrexate is a better substrate for MRP1 through MRP3 than it is for MRP4. This possibility would be consistent with the reduced topologic and amino acid similarity that MRP4 has with MRP1 through MRP3 compared with the structural resemblance that the latter three transporters share with each other (22,24). Differences in substrate selectivity are also suggested by our observation that, in contrast with cells transfected by MRP1–3, MRP4-transfected cells do not display resistance to natural product anticancer agents. In this regard, MRP4 is similar to MRP5, the MRP subfamily member whose topology is most closely related to that of MRP4 (24) and for which resistance to natural product drugs was not observed in transfected HEK293 cells (34).

The observation that MRP4-transfected cells are resistant to the amphipathic anion PMEA indicates that the resistance profile of this transporter extends beyond anticancer agents. In agreement with the drug resistance profile that we describe in this study, MRP4 has recently been reported to be amplified in CEM-r1, a human T-lymphoid cell line selected for high-level resistance to PMEA (38). Consistent with the effects of an efflux pump distinct from Pgp and MRP1, CEM-r1 cells display increased efflux of PMEA but are not cross-resistant to colchicine
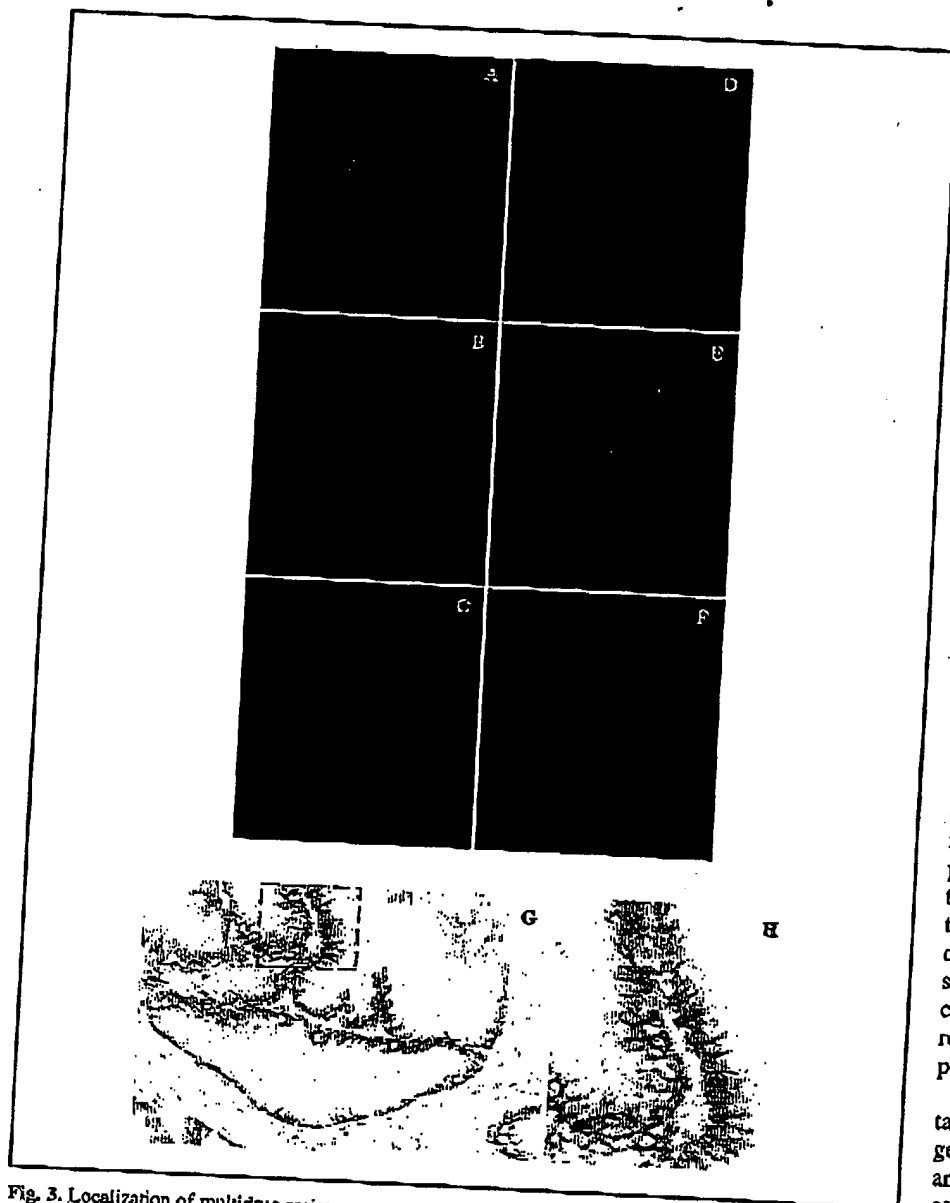
Fig. 3. Localization of multidrug resistance-associated protein (MRP) 4 protein in NIH/MRP4-3 cells (top) and prostate (bottom). Top: MRP4-3 cells (panels A, B, D, and E) and pSRα-transfected NIH3T3 cells (panels C and F) were analyzed by indirect immunofluorescence by use of MRP4 monoclonal antibody as described in the "Materials and Methods" section. Panels A and D—composite image of MRP4-3 cells; panels B and E—horizontal midsections of A and D, respectively; and panels C and F—composite image of control pSRα-transfected cells. Bottom: Immunohistochemical detection of MRP4 in prostate tissue. Immunohistochemistry was performed as described in the "Materials and Methods" section. Panel A: section of human prostate showing MRP4 staining in tubuloacinar cells (original magnification ×100). Panel B: enlargement of the area indicated by broken lines in A (original magnification ×450).

ter cell line exhibits cross-resistance to nucleoside analogues (AZT and 3TC), for which we did not observe resistance in MRP4-transfected cells. Alternatively, high expression levels of MRP4 may be required to detect resistance to AZT and 3TC.

PMEA is an acyclic nucleoside phosphonate that acts as a stable monophosphate analogue of adenosine monophosphate and deoxyadenosine monophosphate, exhibits activity against a variety of DNA viruses and retroviruses, and has clinical activity against human immunodeficiency virus-1 (40). Nucleoside analogues represent one of the two major classes of drugs used in treating human immunodeficiency virus-infected patients, and resistance to this class of agents is a major clinical problem. Although we did not observe resistance in MRP4-transfected NIH3T3 cells to commonly used nucleoside analogues, such as ddC, d4T, and 3TC, it is possible that increased expression of MRP4 or other MRP subfamily members may play a role in resistance to these or other members of this class of agents. Of interest, another anticancer drug transporter, Pgp, has been implicated in the transport of protease inhibitors (41–44), the second major class of anti-AIDS drugs. While the idea that Pgp or MRP subfamily transporters play a role in clinical resistance to anti-AIDS agents is currently speculative, it is intriguing in that potent pump inhibitors are available.

The capacity of MRP4 to confer resistance to methotrexate and PMEA suggests that it is able to transport glutamate and phosphate conjugates and possibly other amphipathic anions. However, its physiologic functions are currently unknown. The immunohistochemical analysis of MRP4 expression in the prostate may provide some insights into its possible functions. MRP4 was detected primarily in the tubuloacinar cells of this organ and, within these polarized cells, it was localized in the basolateral but not in the apical plasma membranes. This suggests that MRP4 localization in polarized cells is similar to that of human MRP1 and MRP3, both of which have basolateral localization (33,45–47), and distinct from the apical MRP2 transporter (17,48,49). On the basis of the localization in the prostate, we speculate that one function of MRP4 might be to efflux xenobiotics out of prostatic epithelial cells and into the stroma, thus helping to protect prostatic fluid. Given its widespread

and vinblastine (39). The detection of PMEA resistance activity in transfected NIH3T3 cells indicates that amplification of MRP4 in CEM-r1 cells is directly related to the activity of this transporter.

In view of the modest levels of PMEA resistance in MRP4-transfected cells (2.3-fold), the amplification of the MRP4 gene in PMEA-selected cells is perhaps surprising. Potential explanations for the low

levels of resistance in transfected cells are that expression of MRP4 in NIH3T3 transfectants is low or that NIH3T3 fibroblasts are less sensitive to PMEA than are CEM cells, and the impact of MRP4 overexpression is, therefore, more pronounced in the lymphoid cell line. Of course, it is likely that the high level of PMEA resistance (250-fold) in CEM-r1 cells is multifactorial. This may explain why the lat-

tissue distribution *(22,26)*, it is likely that MRP4 also represents one f several organic anion transporters that were defined previously in biochemical studies by the use of membrane vesicles prepared from a variety of cells *(50,51)*. Further characterization of the substrate selectivity of MRP4 should provide additional insights into its physiologic functions.

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